Cell Adhesion and Communication, 1998, Vol 5, pp. 257–271
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Localisation of a Novel Adhesion Blocking Epitope on the Human β_1 Integrin Chain

HEYU NI[b†] and JOHN A. WILKINS[*a,c]

[a]*Departments of Internal Medicine* [b]*Immunology* [c]*Medical Microbiology University of Manitoba*

(Received 28 May 1997; Revised 16 July 1997; In final form 16 July 1997)

Members of the β_1 integrin family mediate cellular adherence to a wide range of extracellular and cell surface associated ligands. Conformational changes have been shown to be associated with integrin activation and ligand binding. Some studies suggest that there may be a restricted region of the β_1 integrin that serves as the target for regulatory antibodies which can inhibit or stimulate integrin function. Here we identify an inhibitory epitope that is located at a distinct sight from that suggested for other inhibitory antibodies. Three different adhesion blocking antibodies, JB1A, C30B, and D11B bind to a peptide corresponding to residues 82-87 of the mature β_1 chain. Mn^{++} inhibited the binding of JB1A to purified β_1 integrin. In contrast the binding of several other antibodies to β_1 were not influenced by these conditions. JB1A binding to purified peptide was also inhibited by Mn^{++} suggesting that it related to interference with the antibody function rather than a cation dependent change in the epitope. Our data 1) directly demonstrates the peptide sequence recognised by three adhesion blocking antibodies to the human β_1 integrin chain 2) identifies a novel epitope located at residues 82-87, distinct from that of previously described regulatory epitopes 3) characterises a Mn^{++} sensitive antibody integrin interaction. Collectively, these results indicate the existence of multiple regulatory sites on the β_1 integrin molecule.

Keywords: β_1 Integrin, blocking, epitope, human

INTRODUCTION

The integrins represent one of the major families of adhesion structures expressed on eukaryotic cells [17]. They mediate interactions with components of the extracellular matrix [31], cell surface proteins [4] and infectious agents [18]. Ligand contact can also lead to phenotypic changes in the cells which express them such as the induction of proliferation [21,28], the generation of apoptotic signals [14,41] or the activation of gene expression [37,40]. Integrin dependent activation of a number of signalling pathways such as phospholipid metabolism [7], Ca^{++} and protein phosphorylation [25,26] have also been

*Corresponding author. Rheumatic Diseases Unit Research Laboratory, RRQ14, 800 Sherbrook Street, Winnipeg, Manitoba R3A 1M4 Canada. Ph. (204) 787-7021; Fax: (204) 787-2420; Email: jwilkin@cc.umanitoba.ca

observed. Thus substrate interactions via integrins can provide both positional and response pattern cues to cells which express them.

The integrins are heterodimeric transmembrane proteins in which each subunit consists of a large extracellular domain, a single membrane spanning region and usually a short cytoplasmic region [16]. The α β receptor pair defines the ligand binding specificity of each integrin. Thus the association of one of several α chains with a given β chain leads to the final specificity of the receptor complex. While the molecular mechanisms responsible for ligand recognition and specificity are unknown, it is clear that both α and β chains are involved in ligand contact.

Peptides containing Arg-Gly-Asp (RGD), a sequence recognised by many but not all integrins [3,23] or KQAGGDV [19], a sequence from the carboxyl terminus of the γ chain of fibrinogen which is recognised by $\alpha_{IIb}\beta_3$, have been used in crosslinking studies to localise the ligand contact sites of the β_3 integrins [9,30]. The KQAGGV peptide was found to be associated with residues 294-314 of the β_3 chain [9]. While RGD peptides were crosslinked to β_3 regions containing residues (109-171) and (66-203) in $\alpha_{IIb}\beta_3$ [9] and $\alpha_v\beta_3$ [30] respectively. Further support for the potential involvement of this region in ligand binding derives from the observations in $\alpha_{IIb}\beta_3$ that antibodies that react near this region block integrin binding to fibrinogen [1]. A naturally occurring point mutation in β_3 (119 D → Y) or site directed mutagenesis of homologous residue of the β_1 (130 D → Y) chain resulted in the loss of ligand binding potential [20,35]. More recently it has been demonstrated that this region of the β_3 chain is involved in both ligand and cation binding [10]. This may serve to explain some aspects of cation dependency of integrin function.

The overall amino acid homology of the β subunits (45-50%) as well as the presence of selected areas with very high levels of homology (80%) in β subunits suggests that there may be considerable similarity in the regulation and binding mechanisms of the different integrins [15]. However, there is relatively little known about these aspects of the functioning of non-β_3 integrins. Epitope mapping studies of the β_1 integrins using interspecies β_1 chimeras have provided some insight as to the possible locations of inhibitory and regulatory epitopes [27,36]. In one case the expression of the epitopes recognised by several regulatory antibodies were found to be sensitive to the amino acid changes in residues 207-218 of the β_1 subunit [36]. This has lead to suggestions that a single region may be the target of all known regulatory antibodies to the human β_1 integrin chain.

The present study identifies and localises a novel epitope that is recognised by several antibodies to the β_1 chain that block adhesion. This epitope is distinct from previously described regulatory sites. Evidence is also presented for a Mn^{++}-sensitive interaction of one of these antibodies.

MATERIALS AND METHODS

Materials

Unless otherwise indicated all chemicals were purchased from Sigma Chemicals. Media, fetal bovine serum, and fibronectin were obtained from Gibco Life Sciences. Custom synthesised peptides were purchased from Chiron Mimotopes Peptide Systems, CA and from Research Genetics, AL. The purity of all peptides was greater than 85%.

Monoclonal Antibodies

The antibodies JB1A [33], JB1 [6] 3S3 [11] and B3B11 [39] have been described previously. Antibodies, AP-138, and AIIB2 [5] were provided by Dr. Andrew Shaw, Cross Cancer Research Institute, Edmonton, AB and Dr. Caroline Damsky, Dept. of Anatomy, UCSF respectively. A summary of the properties and specificities of the antibodies used in this study is provided in Table I. The specificities of the antibodies for β_1 were confirmed as previously described using CHO cells transfected with the human β_1 gene [39].

TABLE I Properties of Antibodies used in this Study

Antibody	IP[a]	Blot[b]	Activity[c]	Ref.
JB1	+	−	weak stim.	[6]
JB1A	+	+	inh.	[33]
C30B	+	+	inh.	(This study)
D11B	+	+	inh.	(This study)
3S3	+	−	inh.,agg.	[29]
AP-138	+	−	inh.,agg.	−
AIIB2	+	−	inh.,agg.	[5]
B3B11	+	+	stim.	[39]

[a]ability to immunoprecipitate β_1; [b]reaction of antibodies with reduced β_1 on immunoblot; [c]effects of antibodies on cellular adherence to type I collagen and fibronectin; inh.-inhibits adherence; stim.-stimulates adherence; agg.-induces homotypic aggregation of Jurkat cells.

Cells and Culture

The human lymphoid cell lines Jurkat and IM9 (ATTC) was grown in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum.

Cell Binding Assay

Non-tissue culture treated microtiter wells were coated with purified plasma fibronectin, 5μg/ml (Gibco Life sciences) or collagen, 20 μg/ml, as previously described [38]. The wells were washed and blocked with 1% BSA in RPMI.

Cells were normally suspended in RPMI-1640. However for the Mn^{++} studies, they were resuspended in Puck's saline alone or with the indicated cation concentration and then added to fibronectin or BSA coated wells (2×10^5/well) and incubated for 60 minutes at 37°C. The non-adherent cells were removed by centrifugation of the inverted plates for 5 minutes at 70 × g and the supernatants were removed. The adherent cells were fixed and stained for 30 minutes with 0.5% crystal violet in a 30% solution of methanol in water. The plates were washed with water to remove unbound dye and air dried. The residual dye was solubilised in methanol and the absorbance at 550 nm was determined. Binding to collagen was induced in Jurkat cells by stimulating the cells with PMA, 20 ng/ml, for 30 minutes prior to assaying for adherence [38]. In all assays the adherence to BSA was subtracted from the values obtained for the fibronectin coated wells. All experiments were performed at least three times in sextuplicate. All values were within 15% of the mean.

Epitope Library Production and Screening

Libraries were constructed using the NovaTope system (Novagen Inc., Wisconsin) according to the supplier's instructions. The method based on the use of modified pET expression vectors [32] consisted of digesting pFnRβ with DNAse I in the presence of Mn^{++} and size fractionating the random fragments by electrophoresis in 1.2% agarose gels. The 50-150 bp or 150-300 bp fragments were flush ended with T4 DNA polymerase, single dA tailed and ligated into the EcoR V site of the pTOPE-1b (+) plasmid which had been tailed with single 3'dT residues. Novablue (DE3) cells were transformed with the plasmid, and colonies were immunoscreened with a panel of anti-β_1 monoclonals and an alkaline phosphatase conjugated rabbit anti-mouse immunoglobulin. Positive colonies were subcloned and examined for reactivity with the antibodies. The inserts from individual clones were sequenced using a T7 gene 10 primer.

Random Phage Peptide Display Library

An M13 phage based random peptide display library, CMCC #3858, was provided by Chiron Corporation. In this library random 15mer peptides are displayed on the phage, M13LP67, as a gene III fusion proteins [8].

Positive clones were selected by incubating 10^{10} pfu in 1 ml of PBS with 1μg of biotinylated JB1A for 15 minutes with shaking after which 20μl of streptavidin conjugated magnetic beads (Dynal Inc., NY) were added to the phage antibody mixture and incubated for an additional 30 minutes. The beads were magnetically collected and washed 8 times, 1 ml per wash, with PBS containing 0.1% BSA, 0.5% Tween 20. The phage were eluted from the beads in 100μl of 0.1M glycine-HCl, pH2.2, and neutralised with 9μl of 2M Tris pH 8.0. The eluted phage were amplified in competent MV1190 cells on LB agar plates. The phage were purified by PEG NaCl precipitation and tittered [29].

The sequences of the inserts were determined using double stranded DNA sequencing system (BRL/GIBCO) and the oligonucleotide primer ACAGACAGCCCTCATAGTTAGCG [8].

Purification of β_1 Integrin

Integrin was isolated from human placenta using a modification of the method described by Smith and Cheresh [30]. Briefly, 300g of washed placenta was homogenized in 300 ml of 50 mM n-octylglucopyranoside in 25 mM Tris pH 7.6, 150 mM NaCl, 2 mM $CaCl_2$ and 1 mM PMSF. The homogenate was centrifuged 10,000 × g for 1 hr at 4°C after which the supernatant was collected and passed sequentially through an ovalbumin sepharose 4B and a JB1A sepharose 4B column at a rate of 1 ml/minute. The column was washed sequentially with 20 column volumes each of 1) 0.1% NP-40 in 25 mM Tris pH 7.6, 150 mM NaCl, 2 mM $CaCl_2$, 2) 0.1% NP-40 in 0.01 M sodium acetate buffer pH 4.5. The β_1 integrin was eluted from the JB1A column in 0.1% NP-40 10 mM sodium acetate buffer pH 3.6 and 3 ml fractions were collected into tubes containing 0.5 ml of 3M Tris pH 8.8.

The purity of the fractions were assessed by SDS-PAGE and Coomassie blue staining. The fractions containing β_1 also had a mixture of the associated α chains. However, the α and β_1 chains collectively represented greater than 85% of the total stained proteins. The presence of β_1 in the fractions was confirmed by western blot with JB1A and B3B11.

Antibody Binding to Peptides and Purified β_1

For capture of biotinylated peptides on microtiter trays, avidin was suspended at 5 μg/ml in water and allowed to dry overnight, 0.5μg/well, in Nunc Maxisorb plates. The plates were washed three times with 0.5% Tween 20 in TBS and blocked for two hours at room temperature with 1% BSA in TBS. Biotinylated peptides, 100 pM, were added to each well, washed and incubated with the indicated monoclonal antibody at 150 ng/ml after which the binding was quantitated using an alkaline phosphatase conjugated rabbit anti-mouse IgG and substrate.

In the case of nonbiotinylated peptides, they were dissolved in water and added to Nunc Maxisorb plates at 100 pM each/well and allowed to dry overnight. Following three washes with TBS 0.5% Tween 20, the indicated antibodies were added at 5 μg/ml and the ELISA performed as described above.

Cation Effects on Antibody Binding to peptides and purified β1 integrins were assessed by mixing the antibodies with the indicated cations in Puck's saline. The binding of the antibodies to peptides and purified β_1 were then quantitated by an ELISA as described above.

Peptide Blocking of Antibody Binding to Purified β_1 Integrin

The indicated antibodies (150 ng/ml) were pre-incubated with the indicated concentrations of peptide for 18 hours at 4°C. The antibodies were then added to Nunc Maxisorb plates that had been precoated with affinity purified β_1 integrin. The level of binding was quantitated using an alkaline phosphatase conjugated anti-mouse IgG as described above. The experiments were carried out three times with triplicates in each assay.

Flow Cytometry

Washed cells were resuspended in Puck's saline with the indicated divalent cations and saturating concentrations of antibody for 30 minutes. The cells were washed three times in the corresponding buffer and reacted with FITC labelled goat anti-mouse IgG. The cells were washed and the level of staining was determined using a BD FACScaliber. Gating was on live cells and 10,000 events were analysed for each sample. Representative data is provided for one of three replicate experiments.

RESULTS

Characterisation of Antibodies

The antibodies used in the present study were selected for their abilities to block integrin dependent adher-

ence of Jurkat cells. As predicted from previous studies, the antibodies AIIB2, JB1A, and 3S3 blocked Jurkat binding to fibronectin [5,11,33] (Figure 1). AP-138 also caused almost complete inhibition of adherence. The antibodies D11B and JB1A inhibited adherence 80-85%, while C30B was consistently observed to cause a lower level of inhibition that varied between 30-50% inhibition in different experiments. The specificity of the antibody effects was demonstrated by the fact that another antibody to β_1, B3B11, [39] did not interfere with cell binding (Figure 1).

Jurkat cells displayed a low level of $\alpha_2\beta_1$ dependent adherence to collagen that could be enhanced by treatment with the phorbol ester PMA or with stimulatory antibodies to the α_2 or the β_1 integrin chains [34,38,39]. The antibodies 3S3, AP-138, JB1A, and AIIB2 caused an 85%-90% inhibition of adherence of PMA treated cells to collagen (Figure 1). The adherence was also markedly inhibited (i.e. >70%) by C30B and D11B. As previously reported, B3B11 further enhanced the binding of Jurkat cells to collagen [34]. These results indicated that the antibodies JB1A, 3S3, AIIB2, AP-138, C30B and D11B

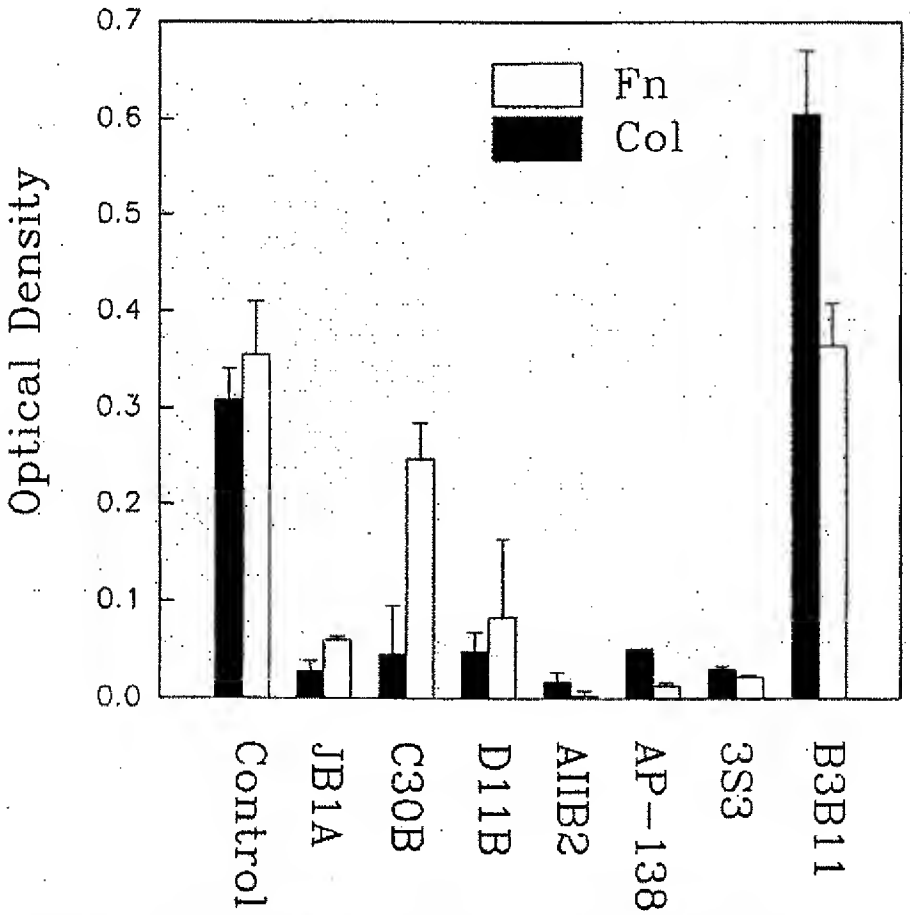

FIGURE 1 The effects of antibodies to β_1 on the adherence of Jurkat cells to fibronectin or collagen. Adherence to collagen was assessed following PMA stimulation of the cells as described in Materials and Methods. Note the anti-β_1 monoclonal, B3B11, stimulates adherence to collagen as previously described [39].

could inhibit β_1 integrin mediated adherence to both collagen and fibronectin further supporting the β_1 specificity of these antibodies.

Identification of the JB1A Binding Region of β_1 Integrin

Preliminary studies had shown that JB1A, C30B and D11B reacted with β_1 integrin chains in western blots under reducing conditions, while AIIB2, 3S3, and AP-138 did not. These results suggested that the former group detected linear epitopes on the β_1 molecule. Based on this assumption, a fusion library containing 50-150 bp inserts of the β_1 cDNA was constructed in an effort to localise the epitopes recognised by these antibodies.

Two clones, A and C, were found to react with JB1A, C30B and D11B but not with the other inhibitory antibodies. The predicted amino acid sequences of the inserts of these clones indicated that they coded for amino acid residues that corresponded to positions clone A, 55-93, and clone C, 61-105 of the mature β_1 (Figure 2). These results suggested that the JB1A, C30B and D11B epitopes were located at positions 61-93 of the mature β_1 chain. However, it was not possible with this strategy to determine the exact location of the epitope recognised by this antibody.

As a complementary approach to defining the JB1A epitope, a random 15mer peptide phage display library was screened for possible mimetics of the natural epitope recognised by the antibodies. Phage were selected using biotinylated JB1A and streptavidin dynabeads. The recovery rose from 10^{-6} to 10^{-1} of initial input (i.e. 10^{10} phage) respectively, for the first and third rounds of selection. Nineteen plaques were randomly selected from the third round for further characterisation. A comparison of the predicted amino acid sequences of the clones indicated that there was consensus TxxKLK in seven clones (Figure 3). An additional five had a related sequence S/GxxKLK. Three other clones contained a common sequence of TxxKLR where a semiconservative substitution of arginine for lysine had occurred. A comparison with the β_1 amino acid sequence suggested that the consensus sequence TxxKLK approximated that of β_1 residues 82-87 TAEKLK. This sequence was also present in both of the fusion protein clones, A and C, isolated from the β_1 epitope library. Thus lending further support to this sequence being the epitope recognised by these antibodies. A minor consensus sequence TxxKLR was also observed in three clones. This sequence approximates that of β_1 residues 179-184 (TPAKLR).

The antibodies JB1A, D11B and C30B were each found to react with the peptide biotin-SGSGTAEKLK while they displayed little reactivity to the peptide SGSGTPAKLR, implying that the former was the epitope recognised by the antibody (Figure 4). In contrast B3B11 which recognises a β_1 epitope at

Clone A

^{55}CPPDDIENPRGSKDIKKNKNVTNRSKGTAEKLKPEDIHQ^{93}

Clone C

^{61}ENPRGSKDIKKNKNVTNRSKGTAEKLKPEDIHQIQPQQLVLRLRS^{105}

FIGURE 2 The predicted amino acid sequences of the two JB1A, C30B, D11B reactive clones, A and C, isolated from the Novatope based β_1 epitope libraries. The superscripts indicate the residue positions of the mature human β_1 integrin. The letters in bold identify the common sequence between the two clones.

Predicted Amino Acid Sequences of JB1A Phage Isolates

Clone No.	Predicted Amino Acid Sequence
1	STS F **KLKHPPTTLSP**
4,7,9,14	QSSTWA**KLKNTLIST**
5	LLARPSSTSH **KLKWQ**
19	LPRNTAY **KLKNSIPS**
15	LFQQPFASAD **KLKPI**
8,11	LSGTS **KLKFWHETSH**
12	MSTHTERYGSM **KLKS**
18	PNHGSQ **KLKNWSLHT**
Predicted Consensus Sequence	**S/T x xKLK**
Corresponding β_1 Sequence	**T AEKLK**
13	MMTIYSHAT **TGKLRS**
3	NVHLPHAT **SSKLRSS**
6	FFKHDSTTC**KLRSCH**
Minor Consensus Sequence	**Tx xKLR**
Corresponding β_1 Sequence	**TPAKLR**
10	LQLSSHFSGTTQ**RLK**
16	PHSERLGTI D**KFLKP**
2,17	QLQSPCKTRD**KLLFC**

βI-Domain

FIGURE 3 The predicted amino acid sequences of the pIII inserts of JB1A isolates from the random peptide phage display library, CMCC#3858. The consensus residues are highlighted in bold. The major consensus sequence is provided with the corresponding predicted region of the mature β_1. A minor consensus sequence identified by clones 3, 6, and 13 is also provided.

residues 657-670 did not bind to either of these peptides [39]). The specificity of this peptide was further confirmed by its concentration dependent inhibition of JB1A, C30B, and D11B binding to purified β_1 (Figure 5). The binding of B3B11 to the purified β_1 integrin was not influenced by the 82-87 peptide under the same conditions. Similarly the peptide 82-87 did not influence the binding of several other inhibitory antibodies, AIIB2, AP-138, or 3S3 to the purified β_1 implying that they detected different epitopes (Figure 6). Thus a peptide corresponding to residues 82-87 of the β_1 integrin specifically inhibited binding of the three adhesion blocking antibodies JB1A, C30B, and D11B.

The Effects of Mn^{++} on JB1A Binding to β_1 Integrin

During the course of studies on the Mn^{++} induced adherence of IM9 cells to fibronectin it was noted that

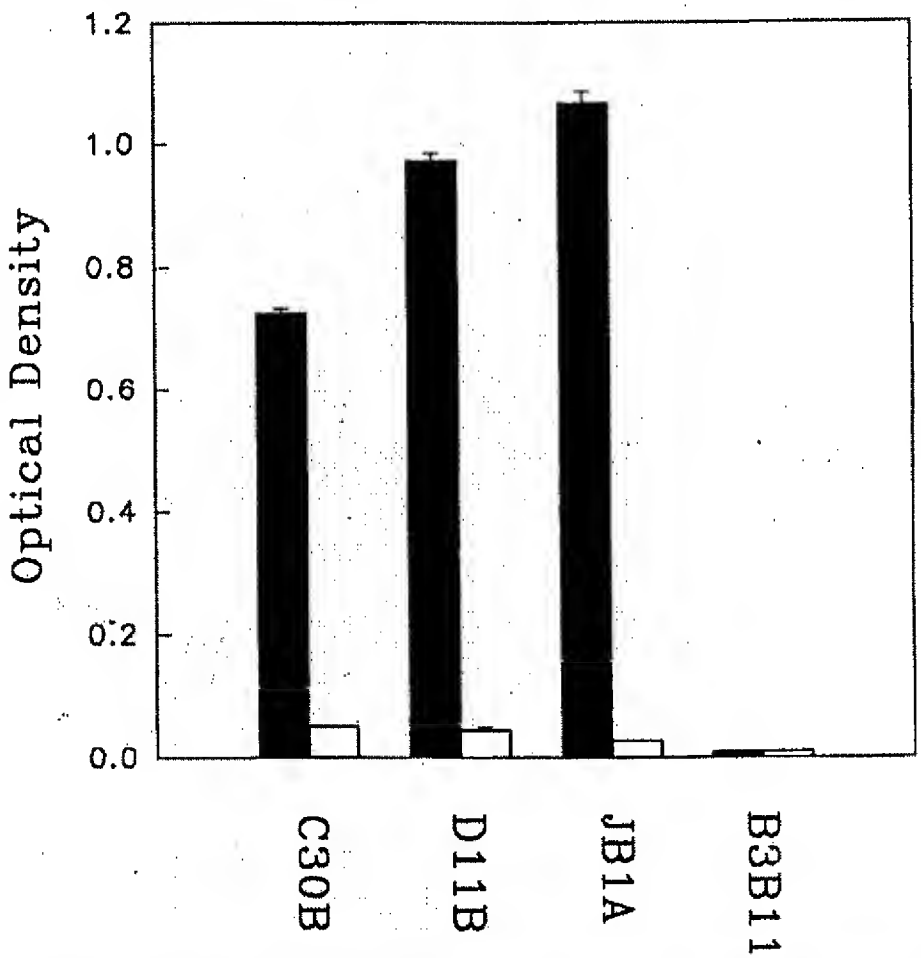

FIGURE 4 The binding of anti-β_1 monoclonal antibodies to peptides containing the predicted JB1A epitopes. The binding of JB1A, C30B, D11B, and B3B11 to the peptides, TAEKLK (82-87) (solid bar), and TPAKLR (179-184) (open bar) identified respectively by the major and minor consensus sequences in the random peptide phage library isolates. Antibody B3B11 has previously been shown to bind to a peptide corresponding to residues 657-670 [39].

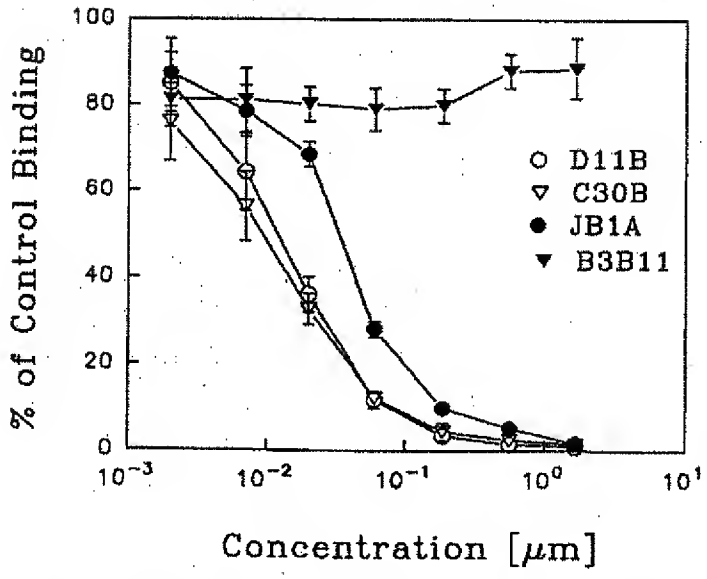

FIGURE 5 The effects of β_1 peptide 82-87 on antibody binding to purified β_1 integrin. Antibodies were mixed with the indicated concentrations of peptide, incubated and assessed for their residual binding to immobilized purified β_1 integrin. The antibodies JB1A, C30B, and D11B were inhibited from binding to the integrin, in contrast B3B11 binding was not influenced.

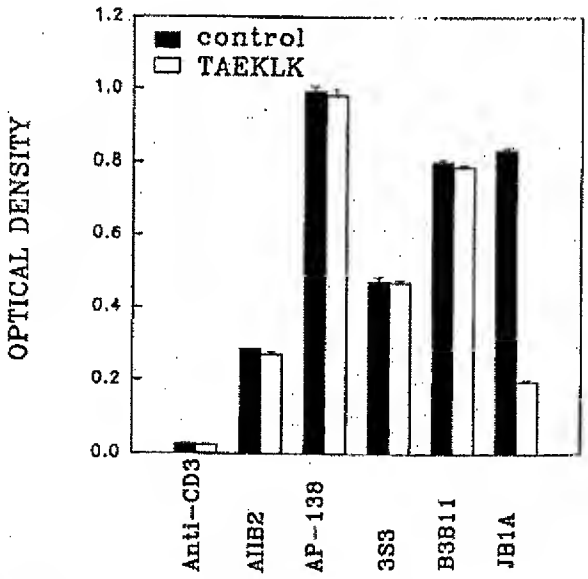

FIGURE 6 A comparison of the effects of β_1 peptide 82-87 on the binding of other antibodies to purified β_1. Antibodies to β_1 were incubated with the peptide TAEKLK and assessed for binding to purified β_1. The anti-CD3 monoclonal was included as a specificity control for antibody binding to integrin.

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there was a marked reduction in JB1A binding to cell associated β_1 in the presence of 8 mM Mn^{++} (Figure 7). Under identical conditions the binding of other antibodies to β_1, C30B, D11B, and 3S3 were not influenced (Figure 7). These effects appeared to be Mn^{++} specific as neither Ca^{++} nor Mg^{++} influenced antibody binding at similar concentrations (data not shown). The inhibition of antibody binding was concentration dependent with maximal inhibition observed at 8 mM of Mn^{++}, the highest concentration tested. These same conditions did not influence 3S3 staining of the IM9 cells (Figure 8). Thus it appeared that this effect was Mn^{++} dependent and specific for JB1A binding.

In order to address directly the mechanism of action of Mn^{++} on JB1A binding, the effects of Mn^{++} on the binding of B3B11 and JB1A purified β_1 and to the β_1 peptide 82-87 were compared. The binding to either of these antigens by JB1A were inhibited by Mn^{++} in a concentration dependent fashion (Figure 9). Although the effects of Mn^{++} on peptide binding appeared to be detectable at lower concentrations than those observed with purified β_1. The binding of B3B11 to β_1 and to a peptide corresponding to the site of the β_1 epitope (residues 657-670) recognised by this antibody [39] was not influenced by Mn^{++} (Figure 9). Similar to the situation observed with IM9 cells the binding of C30B and D11B to purified β_1 and to

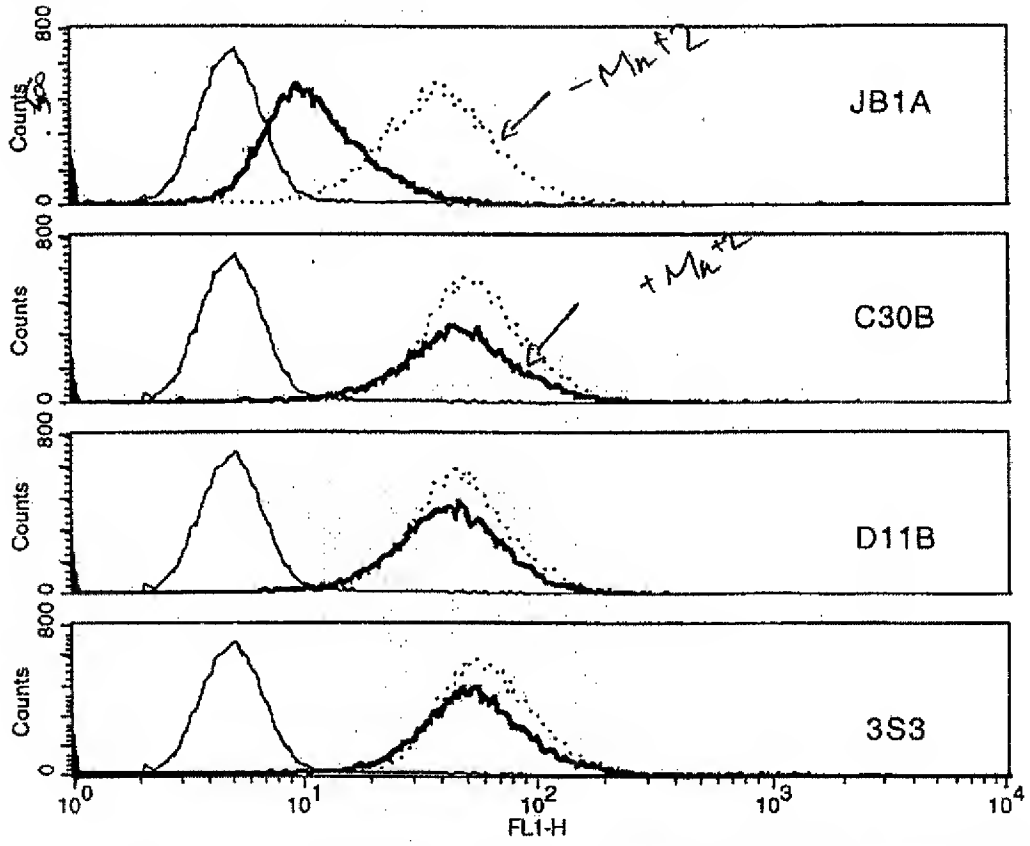

FIGURE 7 The effects of Mn^{++} on JB1A binding to IM9 cells. IM9 cells were washed in Puck's saline and resuspended in the same in absence (dotted line) or presence (solid line) of 8mM Mn^{++}. The cells were stained with the indicated antibodies and analyzed by flow cytometry. The negative control (light solid line) is included with each histogram.

peptides containing the JB1A epitope were unaffected by the presence of Mn^{++} (Figure 10). Collectively these results would seem to indicate that these effects were specific for JB1A. They would also seem to suggest that the Mn^{++} dependent inhibition of JB1A binding relates to a direct effect on the antibody binding to the integrin rather than to alterations of the target epitope on the β_1 chain.

DISCUSSION

The significant results of the present study are: 1) The direct demonstration of the peptide sequence recognised by three adhesion blocking antibodies to the human β_1 integrin chain; 2) The identification of a novel epitope location, distinct from that of previously described regulatory epitopes; and 3) The

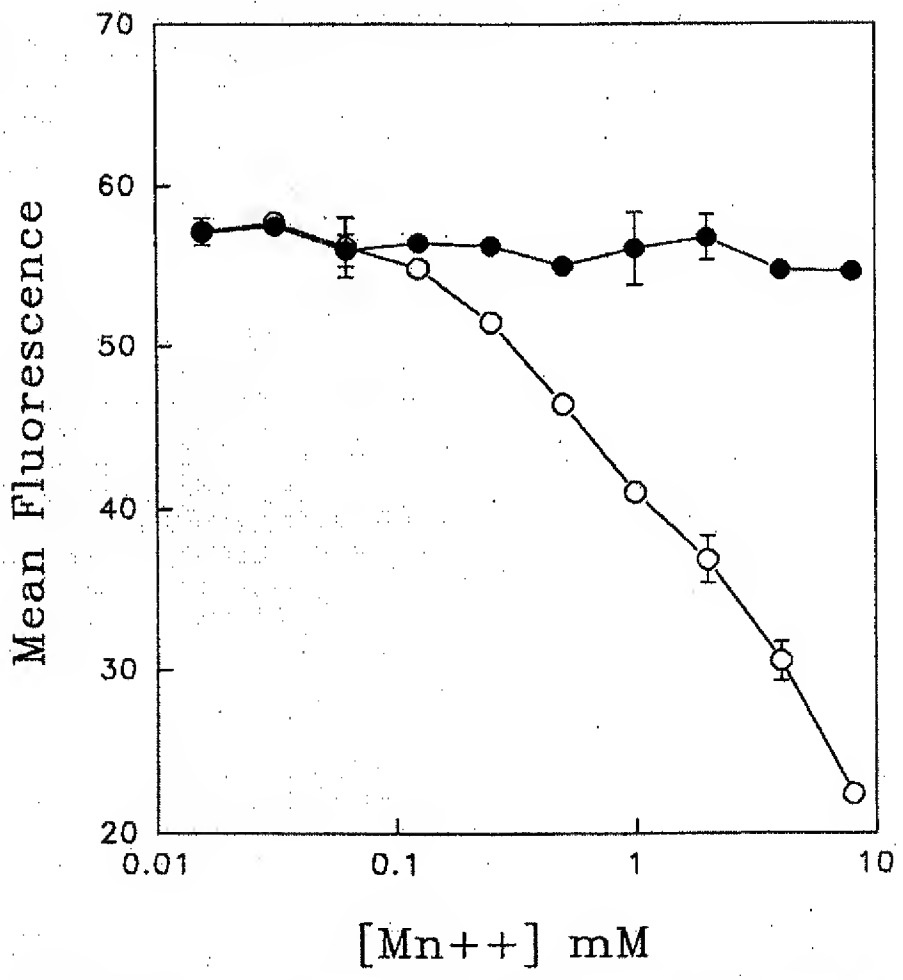

FIGURE 8 The concentration dependence of Mn^{++} mediated inhibition of JB1A binding. IM9 cells were pretreated with the indicated concentration of Mn^{++} and reacted with either JB1A (open circles) or 3S3 (solid circles) and the mean fluorescence intensity of each sample was determined by flow cytometry.

characterisation of a Mn^{++} sensitive antibody integrin interaction.

The epitope recognised by JB1A, C30B and D11B contains residues 82-87 of the human β_1 integrin chain. This conclusion is based on several pieces of evidence. Each of these antibodies binds to fusion proteins that contain fragments of β_1 that span this region of the integrin. A decapeptide, SGSGTAEKLK, containing residues 82-87 is selectively bound by these three antibodies. The binding of these same antibodies to purified β_1 is specifically inhibited by this peptide.

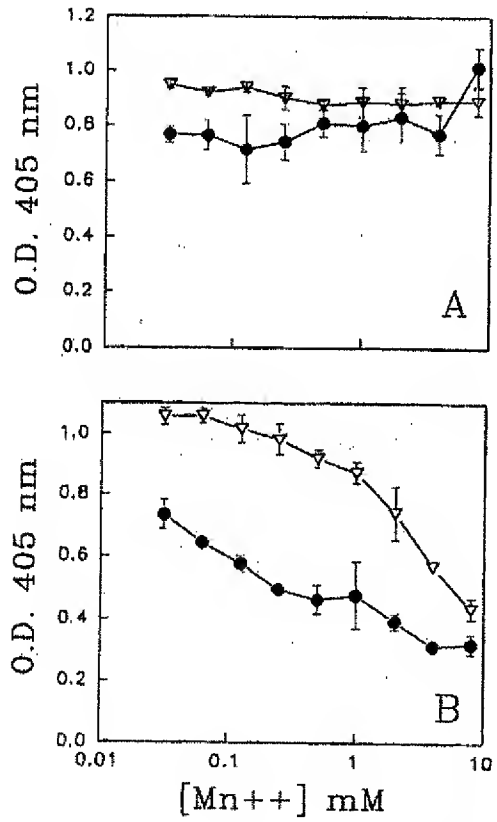

FIGURE 9 The effects of Mn^{++} on antibody binding to purified β_1 and peptides. (A) B3B11 or (B) JB1A were mixed with the indicated concentrations of Mn^{++} and assessed for their binding to β_1 (open triangles) or β_1 peptides (solid circles) 658-666 for B3B11 (A) or 80-90 for JB1A (B).

The location of the JB1A epitope appears to be distinct from that described for a number of other adhesion regulating antibodies [36]. Takada and Puzon reported that residues 207-218 of the β_1 subunit were critical for the binding of a large number of integrin inhibiting or activating antibodies. This sequence is located between two highly conserved regions that are homologous to the putative ligand binding site of β_3 [9,30]. The 207-218 region is also predicted to be part of a β bend in the integrin chain. Antibodies to this region might then be expected to influence integrin function as they could either induce or prevent the appropriate conformational changes involved in the acquisition of ligand binding potential. Shih et. al. also used interspecies chimeric integrins to map β_1 epitopes [27]. In their case, there appeared to be a differential localisation of regulatory epitopes with inhibitory antibodies recognising residues in the first 260 residues while other function modifying antibodies were dependent on the membrane proximal and cysteine rich regions of the β_1 chain. Although the results of each of these studies demonstrated quite conclusively that the expression of certain epitopes were dependent on residues in a given region of the integrin, there was no direct evidence that any of the antibodies actually bound to residues within these regions. This situation arises as a consequence of the fact that the majority of antibodies that were examined in these studies appear to detect conformational epitopes. Thus precluding the detailed direct localisation of their corresponding epitopes.

In a previous study, we have shown that there are at least three distinct loci of epitopes that are recognised by antibodies that can stimulate integrin function [39]. This conclusion was based on the results of competitive antibody binding assays and physical mapping of several of these epitopes. A subset of these antibodies was shown to bind to residues located in the membrane proximal region (657-703) of β_1. These data would seem to collectively indicate that there are multiple regions of the β_1 that contain regulatory epitopes. Such an interpretation appears to be compatible with epitope mapping results in the β_3 integrin were multiple regulatory sites have been identified [13].

It is important to keep in mind that the apparently limited number of regulatory epitopes, which have been described to date, may be a reflection of the high levels of sequence identity observed between human and murine β integrins [12]. Thus it might be anticipated that the antibody data represents a minimal estimate of the number of such epitopes, as many potential sites would be expected to be immunologically silent in this species pair.

The location of the JB1A epitope does not correspond to the homologous ligand contact sites proposed for the β_3 chain [1,9,30]. This may indicate that direct inhibition of ligand binding is not the mode of action of this antibody. A recent study with antibody 13, demonstrated that this antibody interfered with integrin ligand binding by a non-competitive mechanism [24]. It was therefore proposed that the inhibition was a result of allosteric changes

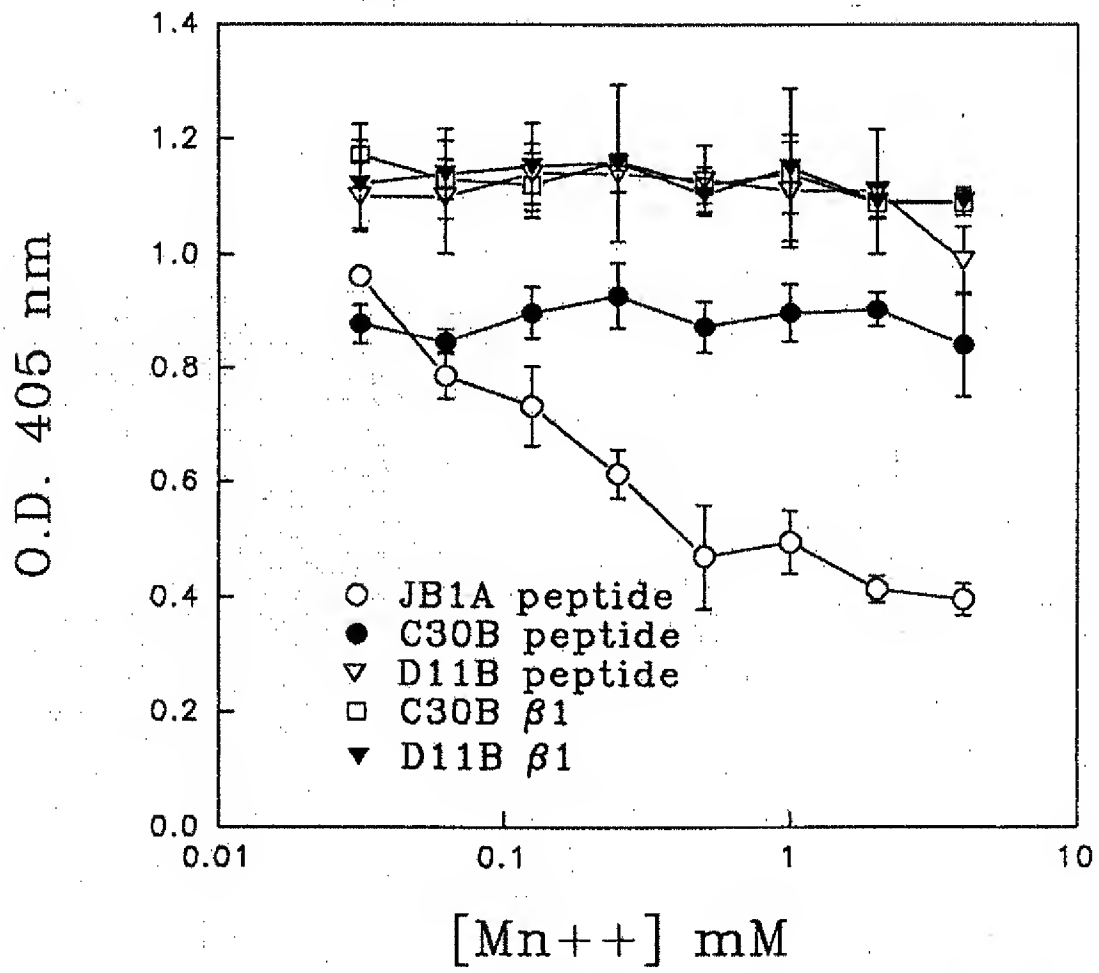

FIGURE 10 A comparison of the effects of Mn^{++} on the binding of JB1A, D11B and C30B to purified β_1 and to β_1 peptide 82-90. Antibodies were mixed with the indicated concentrations of Mn^{++} and assessed for binding to peptide or to β_1.

induced in the β_1 molecule by this antibody. It may be that such a mechanism is operative in the case of the inhibition observed by antibodies of the JB1A group.

An alternative explanation for the antibody inhibition by the JB1A group of antibodies may be that the corresponding epitope is located at a secondary site involved in the stabilisation of ligand contact. Inhibition would then arise from direct steric hindrance of ligand binding. To date there is no evidence to suggest such a role for the JB1A epitope. In fact preliminary studies have failed to demonstrate the direct binding of peptides containing the 82-87 region of the β_1 chain to fibronectin (HN, JW unpublished data). While these results do not rule out a role in ligand binding, they do suggest that any such interactions would be of a low affinity.

The initial observations that JB1A binding to cell associated integrins was Mn^{++} sensitive raised the possibility that there was a relationship between the expression of the JB1A epitope and functional state of the integrin. However, there are several observations that strongly argue against such a relationship. 1) Neither C30B nor D11B, which recognise to the same core epitope as JB1A, were inhibited in their binding to cell associated or purified integrin by Mn^{++}. 2) The binding of JB1A to a biotinylated octapeptide, biotin-SGTAEKLK, or to an undecapeptide, KGTAEKLKPED, was inhibited by Mn^{++}, while those of C30B and D11B are not. Furthermore, preliminary infrared spectroscopic data did not provide any evidence of cation dependent spectral changes (unpublished data JAW, MJ). Thus it seems highly unlikely that the cation effects are a result of conformational changes in the peptide or of interactions between the peptide and cations. There have been a number of antibodies that have been reported to detect cation sensitive epitopes on the integrin β chains [2,13,22]. In some, but not all, of these cases other stimuli have been shown to alter antibody binding implying that conformational changes in the antigen are involved in the altered antibody binding properties. The results with JB1A should serve as a cautionary note regarding the interpretation of cation effects on antibody binding to their antigens. This data also points to the potential value of direct epitope mapping in the resolution of such issues.

Acknowledgements

We thank Dr. Mike Doyle, Chiron Corp. for providing the random peptide phage libraries and advice in their use; Dwayne Stupack and Caixia Shen for their roles in the production and characterisation of several of the monoclonal antibodies; Dr. Guangming Zhong for his advice and assistance with the flow cytometry. This research was supported by grants from the Medical Research Council of Canada, the Canadian Arthritis Society and the Manitoba Health Research Council. Heyu Ni is the recipient of a Manitoba Health research Council Studentship Award.

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